INCORPORATION OF OLIGODEOXYNUCLEOTIDES INTO DNA*

BY MEHRAN GOULIAN

DEPARTMENTS OF MEDICINE AND BIOCHEMISTRY, UNIVERSITY OF CHICAGO,
AND THE ARGONNE CANCER RESEARCH HOSPITAL†

A recent report described a system that used DNA polymerase and poly-nucleotide-joining enzyme for conversion of single-stranded circular DNA to closed duplex circles.[1] There was a requirement in that system for a small amount of the supernatant from a boiled extract of *Escherichia coli*. The requirement for boiled extract was not well understood although its effect appeared to be on replication of the circular template rather than on the subsequent joining reaction. The present study began as an inquiry into the nature and function of the active material in the boiled extract.

This report will describe properties that permit assignment of the activity to oligodeoxynucleotides, and the demonstration that the latter are incorporated into the enzymatic product. A succeeding report will present further studies that support a mechanism for the role of oligomers in the initiation of DNA synthesis by DNA polymerase.[2]

Materials and Methods.—Pancreatic RNase,[3] trypsin, snake venom phosphodiesterase, and calf thymus DNA were purchased from Worthington, and RNase T_1 and Pronase from Calbiochem.

Sucrose gradient sedimentations were carried out at 20° in 3.7-ml vol, with IEC 11 × 55-mm tubes with special adaptors, in either the Spinco SW40 or IEC SB-283 rotors. All gradients were linear 5–20% sucrose in either 0.01 M Tris-HCl (pH 8.1), 0.1 mM EDTA (neutral), or 0.3 M NaOH, 0.7 M NaCl, 1 mM EDTA (alkaline). Fractions were ordinarily collected on 3 MM paper disks and acid-washed in a single batch. However, for gradients containing very large amounts of radioactivity (e.g., Figs. 4–6, below), to prevent cross contamination samples were collected in tubes, acid-precipitated with carrier, and individually washed on glass filters.[4]

Concentration of DNA refers to total nucleotide content unless stated otherwise.

Other materials and methods were as described previously.[1, 4]

Results and Discussion.—*Properties of the activity in crude boiled extract:* Boiled *E. coli* extract enhanced the template activity of circular single-stranded DNA. Synthesis by DNA polymerase in a given period of time with a mixture of circular DNA and boiled extract was greater than the sum of the syntheses obtained with these separately, i.e., with circular DNA alone or with extract alone. However, the increment was small compared to the substantial template activity of the boiled extract, thus making it unsuitable as a basis for assay. Instead, the activity in crude extract was measured by the formation of duplex covalent rings from single-strand rings (Fig. 1). With this assay it was found that the activity was not dialyzable and was precipitable with acid. The active material was stable to proteolytic enzymes, alkali, and RNase, an indication that it was neither protein nor RNA (Table 1). The activity could, however, be destroyed by treatment with *E. coli* exonuclease I, which digests single-stranded DNA,[5] or by the combined action of pancreatic DNase and venom phosphodiesterase (Table 1); this strongly suggested that the active material was a polydeoxynucleotide.
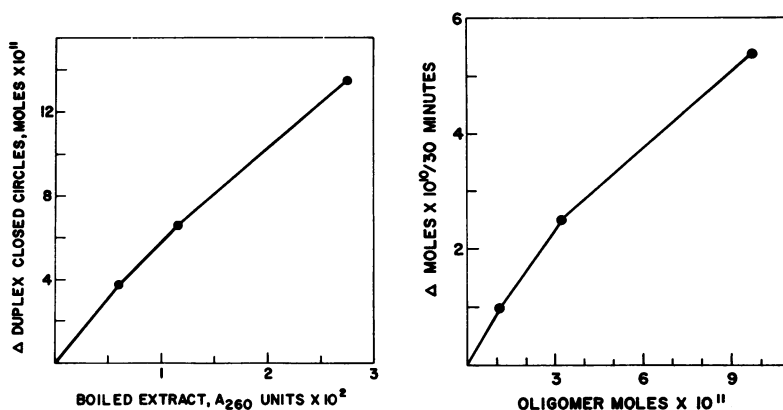
(*Left*) FIG. 1.—Assay of crude boiled extract with the use of replication-joining reaction. The mixture (14 μl) contained 60 μM H^3 ϕX174 DNA, *E. coli* DNA polymerase (400 units/ml), *E. coli* polynucleotide-joining enzyme (0.5 unit/ml), 0.6 mM each of dCTP, dTTP, dATP, and dGTP, 8 μM DPN, 5 mM MgCl$_2$, 20 mM Tris-HCl (pH 7.6), 70 mM KCl, 1 mM β-mercapto-ethanol, albumin (40 μg/ml) and *E. coli* boiled extract in the amounts indicated (1 A$_{260}$ unit = 1 ml with absorbancy of 1, at 260 mμ, in 1-cm path length). Following incubation for 270 m at 25°, the mixture was analyzed by alkaline sucrose gradient sedimentation (35,000 rpm, 55 min; see *Methods*) to determine the amount of duplex covalent circles that were formed. The ordinate (Δ duplex closed circles) was obtained by subtracting the figure for an incubation that contained no boiled extract (14 $\mu\mu$moles).

(*Right*) FIG. 2.—Assay of ability of oligodeoxynucleotides to stimulate replication. Mixture (14 μl) was the same as described in Fig. 1, except for inclusion of α-P^{32}-dATP (20,000 cpm/mμmole), omission of joining enzyme and DPN, reduction of polymerase to 70 units/ml, and replacement of the crude boiled extract by an oligomer mixture (see Fig. 3, below) in the amounts indicated on the abscissa. After incubation for 30 min at 37°, the amount of synthesis was measured as acid-insoluble P^{32} by the glass filter method. The amount of synthesis without added oligomer (50 $\mu\mu$moles) was subtracted to give the figures on the ordinate. In the absence of ϕX174 DNA, there was less than 5 $\mu\mu$moles synthesis obtained with 0.10 mμmole oligomer.

TABLE 1. *Effect of various treatments on the activity in boiled extract.*

Treatment	Activity remaining (Δ duplex closed circles, moles $\times$ 10^{11}/A$_{260}$ unit)
None	5.9
Pronase	4.5
Trypsin	5.6
KOH	5.0
Pancreatic RNase + RNase T$_1$	5.3
E. coli exonuclease I	<0.5
DNase + venom diesterase	<0.5
DNase	64.0

Assay conditions were the same as for Fig. 1. The enzyme treatments were carried out with the extract diluted with 20 mM Tris-HCl (pH 7.6) to approximately A$_{260}$ = 100. The following conditions were used: Pronase, 0.3 mg/ml, 60 min; trypsin, 0.1 mg/ml, 60 min; KOH, 0.3 M, 18 hr; pancreatic RNase, 0.1 mg/ml, plus RNase T$_1$, 20 μg/ml, 60 min; *E. coli* exonuclease I, 500 units/ml, 30 min; pancreatic DNase, 20 μg/ml, plus venom phosphodiesterase, 20 μg/ml, 30 min; pancreatic DNase (alone), 50 μg/ml, 60 min. The latter three mixtures included MgCl$_2$ at 2 mM, and all mixtures were incubated at 37° except KOH which was at 25°. Trypsin was inactivated at 100°, 10 min; inactivation of the other enzymes, except Pronase, was carried out (after addition of EDTA, 5 mM) at 100°, 10 min, followed by treatment with 1 M KOH, 37°, 30 min. Pronase was extracted three times with phenol at 70°, the phenol was removed with ether (seven extractions), and the mixture treated with 0.3 M KOH, 18 hr, 25°. In all cases, KOH was neutralized with HCl.

Activity of oligodeoxynucleotides: Treatment of crude boiled extract with pancreatic DNase, which forms oligonucleotides from DNA,[6] increased the activity tenfold (Table 1).   Purified high-molecular-weight *E. coli* DNA had no detectable activity but after treatment with DNase became highly active.   Untreated calf thymus DNA also had no activity but became active after digestion with pancreatic DNase.   These results indicate that the activity may be ascribed to oligodeoxynucleotides and that there was no specificity as to source of the latter.

Digestion with pancreatic DNase destroyed almost all intrinsic template activity in the boiled extract, making it possible to employ a simpler assay which measured augmentation of the template activity of a single-stranded DNA by the digest (Fig. 2).   The activity measured by this assay was the same as with the replication-joining assay described earlier, as indicated by the parallel results obtained when both assays were performed on samples of different activity.   This assay was used to examine the effect of oligomers on DNA templates other than ϕX174 DNA.   The results indicated that with a given source of oligodeoxynucleotide there was also no strict specificity for template.   Although varying widely in efficiency, oligomers from either *E. coli* or calf thymus stimulated replication of a variety of templates, including denatured DNA from *E. coli*, calf thymus, and T7 and T4 bacteriophages (Table 2).   There was no detectable stimulation of the replication of double-stranded DNA by oligomers.

Oligomers in a wide range of chain lengths were active.   A DNase digest of *E. coli* DNA when chromatographed on Sephadex G-50 showed a distribution of activity from a chain length of approximately four residues to 30–40, near the exclusion limit[7] (Fig. 3).   The latter represents the upper limit found in these digests rather than an upper limit of effective size.   Longer chains were not tested.

The basis for the observed differences in enhancement of template activity with different template oligomer combinations (Table 2) is not clear and could
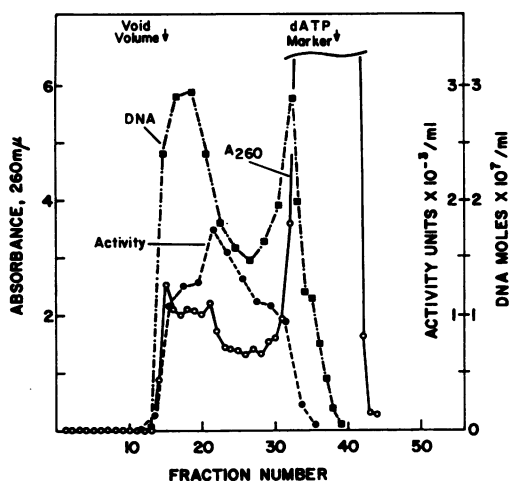

FIG. 3.—Sephadex G-50 chromatography of crude oligodeoxynucleotide mixture. Boiled extract from 3 gm of *E. coli* cell paste[1] was treated with DNase (50 μg/ml, 60 min, 37°) and then extracted three times with phenol at 50°. After the phenol was removed with ether, the extract was treated with 0.3 *M* KOH for 18 hr at 24° to digest RNA and then passed through a column of Sephadex G-50 (0.64 cm² × 90 cm) in 1 *M* KCl, 10 mM Tris-HCl (pH 8.4), 1 mM EDTA. Fraction size was 1.7 ml. DNA (or deoxyribonucleotide) was assayed by the fluorometric method of Kissane and Robbins.[8] Activity (1 unit = Δ 1 mμmole/30 min) of the the fractions was determined by the assay described for Fig. 2. Marker positions for void volume and dATP are indicated by arrows at top.   For experiments requiring oligodeoxynucleotides, active fractions prior to the late rise in absorbancy (e.g., fractions 16–27) were pooled, dialyzed against 1 mM Tris-HCl (pH 8.1), 10 μM EDTA, and concentrated by rotary evaporation.

TABLE 2.   *Comparison of stimulatory effect of two different sources of oligodeoxynucleotides*
*on the template activity of various single-stranded DNA's.*

Source of template DNA	Source of oligodeoxynucleotide	Stimulatory effect (Δ moles product/30 min/moles oligomer)
ϕX174 bacteriophage	*E. coli*	4.0
ϕX174 bacteriophage	Calf thymus	2.5
E. coli	*E. coli*	0.92
E. coli	Calf thymus	0.48
Calf thymus	*E. coli*	0.20
Calf thymus	Calf thymus	0.17
T4 bacteriophage	*E. coli*	0.96
T7 bacteriophage	*E. coli*	0.92

Assay conditions were the same as described for Fig. 2, except for replacement of the ϕX174 DNA (template) by an equivalent amount of heat-denatured DNA from other sources, as indicated. The oligodeoxynucleotide mixture (Fig. 3) was present at concentrations that varied from 7 to 50 μM. All incubations were for 30 min at 37°. The syntheses obtained with two control incubations, one without template and one without oligomer, were subtracted from the synthesis obtained with the complete mixture. This increment in product was divided by the amount of oligomer in the 14-μl mixture to give the figures listed here for comparison.

include trivial factors, e.g., content of small fragments in the template preparation. Homology, as regards source of the template and oligomer, does not appear to be an important factor. It would be expected that oligomers of this size range would include a random mixture of nucleotide sequences, irrespective of DNA source. However, the possibility that among these, some preferred, or even unique, sequence is required for activity cannot be excluded at this time.

Incorporation of oligodeoxynucleotides into the enzymatic product: In homopolymer template systems such as poly dA plus oligo dT, the oligomer serves as primer on the complementary polymer template,[9, 10] and similar mechanisms have also been considered for DNA.[11–13] Since this type of chain initiation implies incorporation of the oligomer into the enzymatic product, the relevance of this mechanism to the present system can be tested directly. P^{32}-labeled oligomers were prepared from P^{32}-labeled *E. coli* DNA with the same procedure that was used to prepare unlabeled oligomer (Fig. 3). When P^{32}-oligomers were employed in the replication-joining reaction with H^3-labeled circular DNA template, there was clear evidence of P^{32} inclusion in the duplex circular product identified by alkaline sucrose gradient sedimentation (Fig. 4). The ratio of P^{32} to H^3 across the peak was constant and was equivalent to 1 atom of labeled phosphorus per 25,000 nucleotide residues in the product strand. Since there are 5,500 residues in the product strand, this is equivalent to 1 atom of phosphorus for every five duplex circular molecules. In some other experiments there was as much as twice this amount of phosphorus (Table 3), but the figure always remained well below 1 atom of phosphorus per DNA molecule.

This very low level of incorporation would at first seem to exclude the hypothesis relating the activity of oligomer to a primer function (see above), and suggest that the P^{32} inclusion represents some form of artifact. One possibility for artifact is contamination of the oligomer mixture with P^{32}-labeled deoxynucleoside triphosphate from the labeled *E. coli* extract. Four reasons can be cited against this: (1) a Sephadex fraction that should have had a reduced contamination with triphosphates gave the same amount of P^{32} in product (Table 3, III B);
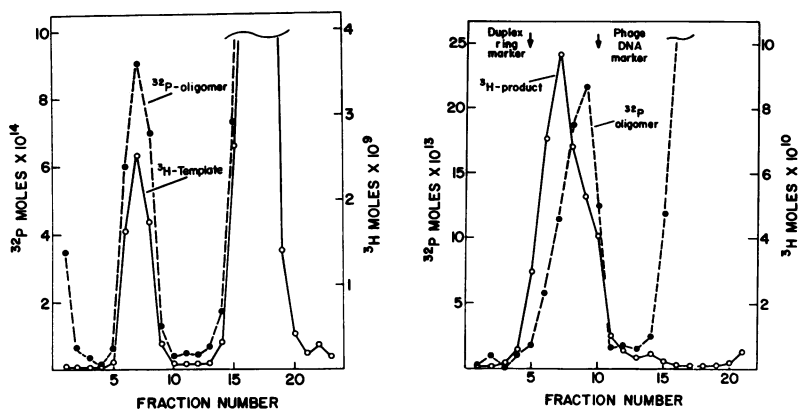
(*Left*) FIG. 4.—Alkaline sucrose gradient sedimentation of duplex closed circles containing P^{32} derived from oligodeoxynucleotides. The incubation mixture (0.42 ml) was the same as described in Fig. 1, except that P^{32}-labeled oligodeoxynucleotides (see text) (6.7 μM, 740 cpm/ $\mu\mu$mole) replaced the boiled extract. Incubation for 270 min at 25° was followed by alkaline sucrose gradient analysis (Fig. 1). Under the conditions used for the latter, the peak of duplex closed circles appeared in fraction 7.

(*Right*) FIG. 5.—Neutral sucrose gradient sedimentation after limited synthesis showing P^{32}-labeled oligodeoxynucleotide in template-product complex. Incubation mixture (0.25 ml) contained 0.3 mM ϕX174 DNA (unlabeled), H^3-dCTP (120 cpm/$\mu\mu$mole), DNA polymerase (100 units/ml), and 11 μM P^{32}-labeled oligomer (330 cpm/$\mu\mu$mole) and was otherwise as described in Fig. 2. After incubation for 5 min at 37°, the reaction was stopped with EDTA (10 mM) and was followed by sedimentation in a neutral sucrose gradient for 420 min at 20°, 30,000 rpm (see *Methods*). Marker positions for ϕX174 phage DNA and partially synthetic ϕX174 duplex closed circles are indicated by arrows at top.

(2) different concentrations of unlabeled triphosphates in the incubation mixture, which would have varied the specific activity of contaminating P^{32}-labeled triphosphates, were associated with the same amount of P^{32} in the product (Table 3, III C); (3) triphosphate had probably been eliminated from the P^{32}-labeled mixture even before the Sephadex step (the *E. coli* cells used as starting material were severely depleted of phosphorus, and persistent activity of the derepressed alkaline phosphatase, which is heat-stable, was indicated by the inability to demonstrate terminal phosphate groups (with bacterial alkaline phosphatase) on the P^{32}-oligomers); and (4) there was inhomogeneous distribution of the P^{32} label in the product chains, to be demonstrated below (Figs. 5 and 6).

A second type of artifact that might have accounted for the labeled phosphorus in the product is mechanical trapping of oligomer in the complex structure that results from alkaline denaturation of the duplex covalent rings.[14] If the trapped P^{32} fragment had a chain length of 40, it would have been present in only one of 200 duplex molecules. Simply from considerations of charge repulsion, this type of trapping would seem extremely unlikely, and it was excluded here by the following experiments. The reaction described for Figure 4 was repeated, and at the end of the incubation period the mixture was divided into two parts. One part was passed over Sephadex G-200 to remove oligomers before alkaline denaturation and alkaline sucrose gradient sedimentation. The other part was directly denatured, in the usual way, with the oligonucleotides still present. The

TABLE 3. *Incorporation of phosphorus from P^{32}-labeled oligodeoxynucleotides into $\phi X174$ duplex circles.*

Experiment		Conversion of phage DNA to duplex (%)	Incorporation (P^{32} per duplex*)
I		17.6	0.46
II		22.1	0.20
III	A. Control	22.1	0.15
	B. Earlier Sephadex pool of oligomer	22.6	0.14
	C. Lower concentration of triphosphates	21.7	0.14
IV	A. Control	16.4	0.23
	B. P^{32}-oligomer removed before denaturation	16.5	0.21
	C. Unlabeled oligomer; replaced by P^{32}-oligomer before denaturation	17.9	<0.01

Incubation mixtures (0.21–0.42 ml) for I, II, III A, and IV A and B were as described for Fig. 1, except for replacement of the boiled extract by 21 μM P^{32}-labeled oligomer (800 cpm/$\mu\mu$mole) (see text). After 270 min at 24°, the reaction was terminated with EDTA (10 mM). In III B, the P^{32}-labeled oligomer was the first half of the Sephadex G-50 pool (Fig. 3) used for the other incubations; III C was the same, except that the concentration of each triphosphate was 0.15 mM instead of 0.6 mM. Details of part IV are given in the text. All mixtures were denatured with NaOH (0.12 M) before centrifugation in alkaline sucrose (see legend to Fig. 1).
* Refers to ratio of moles labeled phosphorus to moles duplex molecules (not residues).

P^{32} included in the product was the same in both cases (Table 3, IV A and B). An additional reaction was carried out with an equivalent amount of unlabeled oligomers, which were removed by Sephadex treatment at the end of the incubation. The same amount of P^{32}-labeled oligomers were then added, and the mixture was denatured with alkali and sedimented in alkaline sucrose. In this case there was no P^{32} in the product (Table 3, IV C).

It would seem, therefore, that the source of P^{32} in the product was the oligomer and that the association with product was not the result of some form of mechanical trapping. The possibility that the low level of incorporation may have been related to the exonuclease activities of DNA polymerase, now known to be capable of attack at both 3′ and 5′ termini,[15, 16] was underlined by the progressive loss of acid-insoluble P^{32} during the course of the incubation. The prolonged incubations with large excesses of polymerase enzyme were considered to have favored loss of oligomer in the product. Accordingly, short incubations with limiting polymerase levels were used in an attempt to retain more of the fragments in the product. The results of such an incubation, for five minutes at 37°, examined in a neutral sucrose gradient, are shown in Figure 5. A separate alkaline sucrose gradient (not shown) on a portion of the same material indicated that the product had a molecular weight of 700,000, i.e., the circular DNA templates were, on the average, approximately 40 per cent replicated at the time the reaction was stopped. Instead of 1 atom of oligomer-phosphorus in 25,000 residues of product, as in the previous experiment, the ratio here was 1 in 500. In control experiments it was found that omission of either triphosphates, Mg^{++}, or enzyme eliminated the P^{32} peak as well as the product. Thus, under the conditions used here, since synthesis was necessary, simple annealing of oligomer to template does not explain the association of P^{32} with product. This experiment also demonstrates the inhomogeneity in distribution of P^{32} in the product that
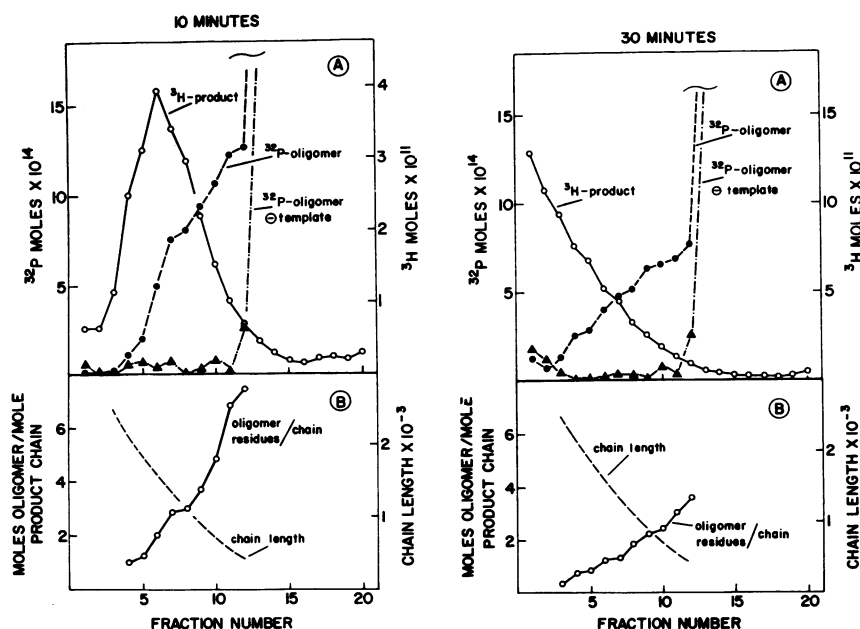
Fig. 6.—Alkaline sucrose gradient sedimentation after limited synthesis showing P^{32}-labeled oligodeoxynucleotide in product. Incubation mixtures (0.025 ml) were the same as for Fig. 5. Incubations were for 10 min and 30 min at 22° and were followed by sedimentation in alkaline sucrose for 650 min at 32,400 rpm. A control incubation and sedimentation, which was carried out in parallel, was identical except for omission of the φX174 (template) DNA. P^{32} of the control (− template) is included above for comparison. (A) includes the P^{32} and H^3 for the complete mixture, as well as the P^{32} of the control (− template) for comparison. For (B), chain length was estimated by the method of Studier;[17] from this was calculated moles oligomer (nucleotide)/mole product chain for each fraction.

was referred to above as evidence against a triphosphate mechanism for the P^{32} incorporation.

In similar experiments, carried out at 22°, sedimentation in alkali showed again the heterogeneous distribution of P^{32} in the product (Fig. 6A). In the shorter incubation (10 minutes) of Figure 6, the product had a molecular weight estimated from sedimentation[17] to be 480,000 at the peak, indicating 30 per cent completion of replication. The ratio of atoms of labeled phosphorus to nucleotide residues in product varied from 1/2000 in fraction 4 to 1/60 in fraction 12. From estimates of molecular weight for these fractions it was calculated that the content of labeled phosphorus atoms per chain varied from 1 in fraction 4 to 7 in fraction 12 (Fig. 6, 10 minutes, B). A sample from the same experiment after incubation for an additional 20 minutes showed that, in spite of the expected increase in amount (and size) of product, the total amount of product-associated P^{32} was actually less than in the earlier sample (approximately 2/3) (Fig. 6, 30 minutes, A). At the later time, the content of labeled phosphorus per chain had fallen to less than 1 in fraction 4 to a maximum of 4 in fraction 12 (Fig. 6, 30 minutes, B). The bulk of the enzymatic product at 30 minutes, now at the bottom of the gradient, had no detectable P^{32} associated with it.

It may be concluded, therefore, that oligodeoxynucleotides underwent direct covalent incorporation into the enzymatic product. The amount of oligomer found associated with product varied greatly with the conditions employed and was greatest in the shortest product chains and after short incubation times. The data may be interpreted to mean that, initially, there was one oligomer associated with one product chain, followed by progressive removal, presumably by the exonuclease activities of DNA polymerase, until little or no oligomer material remained. This then does support a mechanism of initiation by DNA polymerase with oligomer primers, for which additional evidence will be presented in a subsequent report.

Summary.—Oligodeoxynucleotides augment the template activity of single-stranded DNA for *E. coli* DNA polymerase. The effect varies widely in intensity, but there does not appear to be strict specificity in regard to source of oligodeoxynucleotide, source of template DNA, or chain length of oligodeoxynucleotide.

The oligodeoxynucleotides are incorporated into the newly synthesized DNA in covalent union. The data are consistent with the initial inclusion of one oligomer in each product chain and warrant the tentative conclusion that the oligomer serves as primer for the new chain. With continued synthesis the amount of oligomer in the product diminishes progressively, due to the exonuclease activities associated with DNA polymerase, with the result that oligomer is ultimately completely removed from most product strands.

* Supported in part by the Ortho S. A. Sprague Memorial Institute, Chicago.

† Operated by the University of Chicago for the Atomic Energy Commission.

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[3] Abbreviations used are: RNase, ribonuclease; DNase, deoxyribonuclease; dATP, dCTP, dGTP, and dTTP, deoxynucleoside 5'-triphosphates of adenine, cytosine, guanine, and thymine; EDTA, ethylenediaminetetraacetate; DPN, diphosphopyridine nucleotide; Tris, tris-(hydroxymethyl)aminomethane.

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